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The major goal of this project is to constitutively express syngeneic MHC class II molecules (I-A^d), the T cell costimulatory molecules (B7-1, B7-2) or both on murine mammary carcinoma tumor cell lines (410.4 and 66.1) and investigate their ability to induce anti-tumor immunity in the host. Transfectants of both tumor cell lines expressing I-A^d molecules showed significantly slower growth in naive Balb/c mice and formed smaller tumors compared to their wild type counterparts, and in some cases were completely rejected. These transfectants showed unimpaired growth in athymic nude mice. Based on these results, it is suggested that the I-A^d positive tumor cells are able to present tumor antigen(s) to and activate the $CD4^+$ T cells resulting in a moderate tumor immunity in the naive immunocompetant host. Administration of murine recombinant IL-12 while reduced the growth of wild type tumor cells, completely abolished the growth of B7-1 transfectants and prevented metastasis. Multiple injections of irradiated I-A^d positive tumor cells induced protective immunity indicating the possible combination of direct and indirect (cross priming) antigen presentation to the tumor-specific T cells. Using the transfectants coexpressing both I-A^d and B7-1 molecules, the therapeutic value of gene-modified tumor cell vaccine will be investigated.

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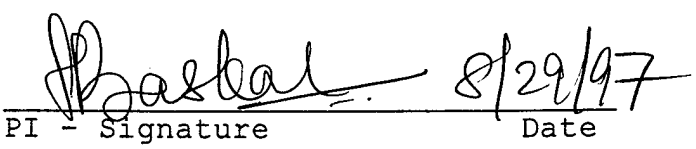

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(5) INTRODUCTION:

The identification and characterization of many tumor antigens and the parallel explosion of knowledge in understanding the cellular and molecular mechanisms of antigen recognition by the immune system have given renewed hopes to envisage immunotherapy as a promising modality to treat certain tumors (1-5). Initial trials of immunotherapy of cancer in general, breast cancer in particular, used rather non-specific immunostimulators ranging from Bacillus Calmette-Guerin (BCG) and levamisole to interferon, interleukins and monoclonal antibodies (mAb) (6-8). The purpose of the present study is to genetically modify the tumor cells so as to facilitate their recognition by the host's tumor-specific T lymphocytes and induce efficient and vigorous anti-tumor immunity. Such gene-modified tumor cells can be used as cellular vaccine to treat human cancers.

As known with nominal antigens, it is reasonable to speculate that anti-tumor immune response will involve recognition of the "tumor antigen" by $CD4^+$ helper T cells (Th) and $CD8^+$ cytotoxic T cells (Tc or CTL) in the context of major histocompatibility (MHC) class II and class I molecules, respectively (3-5). Clonal expansion of these T cells and their subsequent functional maturation is governed by cytokines and other accessory molecules present on antigen presenting cells (APC) (9). Some of these cytokines besides being autocrine growth factors, also enhance the cytotoxicity potency of the effector cells (10, 11).

Cytokines and tumor immunity:

A variety of cytokines including IL-2, IL-4, IL-10, IL-12, TNFa, IFNg and GM-CSF have been used to augment tumor regression *in vivo* (12). In some cases the therapeutically effective concentrations of cytokines are accompanied by toxic side effects. This problem was overcome by cytokine gene therapy, in which tumor cells were transfected with cytokine genes and sufficient amount of cytokine was released at the tumor site with out leading to high systemic levels (13-16). However, the anti-tumor immunity induced was relatively short-lived and the efficacy varied depending on the tumor model under study (17). In some cases a combination of cytokine administration and vaccine tumor cells genetically engineered to express MHC class I, class II and/or costimulatory molecule such as B7-1 has been shown to offer a synergistic effect that was not seen with either procedure alone. Besides, cytokines such as IL-12 and IL-10 have been shown to be antagonistic in promoting the

expansion of Th1 and Th2 type helper T cells, respectively. The impact of such regulatory control on the balance between the Th1 and Th2 cells populations and its effect on tumor immunity is not well understood.

Tumor immunity by expression of MHC class II:

While $CD8^+$ T cells are the major effector cells in killing the target tumor cells, their proliferation and functional maturation requires help from $CD4^+$ T cells. Since MHC class II gene expression is tightly regulated and restricted to professional APCs, majority of the tumor cells do not express MHC class II proteins and can not stimulate $CD4^+$ T cells. As a consequence, in the absence of $CD4^+$ T cell help, the $CD8^+$ T cells are not sufficiently matured or activated. We and others have shown that constitutive expression of MHC class II genes in tumor cells resulted in rejection of the tumor cells by syngeneic host (18-21). Rejection of class II^+ tranfectants resulted in the induction of protective immunity against wild type tumor cells (18-21). These findings strongly suggest that constitutive expression of MHC class II molecules on tumor cells enable them to directly present the tumor peptides to $CD4^+$ tumor-specific Th cells leading to potent anti-tumor immunity (22).

Costimulation of T cells and tumor immunity:

Although the engagement of T cell antigen receptor (TCR) with the antigen/MHC complex is a necessary primary signal for T cell activation, maximal activation of T cells requires additional costimulatory signal(s) (23-25). In fact, in the absence of a costimulatory signal T cells have been shown to enter a state of antigen-specific anergy or deletion (26-28). A variety of cell surface molecules have been shown to deliver costimulatory signals. One of the well studied costimulatory molecules is the B7 family of proteins. Two independently regulated gene products have been identified as members of the B7 family, B7-1 (CD80) and B7-2 (CD86) and these proteins do not share any significant sequence homology (29-31). Both B7-1 and B7-2 have been shown to bind two different receptors on T cells namely CD28 and CTLA-4 (32, 33). Upon receiving the antigen-specific and costimulatory signals both $CD4^+$ and $CD8^+$ T cells undergo further differentiation, enhanced proliferation and secrete elevated levels of cytokines (34-36). We and others have shown that constitutive expression of B7-1 and B7-2 molecules on tumor cells resulted in the rejection of these gene-modified tumor cells and prior injection of $B7^+$ tumors induced

protective immunity against subsequent wild type tumor challenges in syngeneic hosts (37-39). In addition, a synergistic effect was observed when tumor cells were gene modified to express both MHC class II and B7-1 molecules; these cells are potent vaccine capable of inducing complete regression of previously established tumors (40).

Based on these reports, the goal of the present study is to investigate the effect of constitutive expression of MHC class II, costimulatory molecules, B7-1 or B7-2 as well as coexpression of MHC class II and B7-1 molecules in enhancing anti-tumor immunity against murine mammary carcinoma.

Tumor Model:

Murine mammary carcinoma cell lines, 66.1 (metastatic and non-immunogenic) and 410.4 (non-metastatic and weakly immunogenic) were used in the current studies. These tumor cell lines were derived from a parental spontaneous tumor in Balb/c mice (40, 41). They both express normal levels of MHC class I molecules but do not express MHC class II molecules or the costimulatory molecules B7-1 and B7-2. These criteria bring these two tumor cell lines closer to human breast cancer and serve as a model system to study.

The wild type tumor cell lines 410.4/WT and 66.1/WT were transfected with plasmid vectors containing cDNA encoding murine B7-1, B7-2 proteins or cDNAs encoding syngeneic MHC class II alpha and beta polypeptides which form the I-A^d molecule. For the derivation of tumor cells expressing any one of the proteins, cotransfection with plasmid containing neomycin resistance gene was performed in order to allow selection of transfectants from the wild type tumor cells. For the derivation of tumor cells expressing both MHC class II and B7-1, 410.4/Ad and 66.1/Ad clones were transfected with plasmid containing murine B7-1 cDNA and another plasmid containing cDNA for zeocin resistant gene. All transfections were performed using Lipofectine or Lipofectamine as previously described (18, 39). Transfectants were grown in medium containing predetermined concentrations of the selection drug(s) G418 or G418 plus zeocin, depending on the transfectants. Surface expression of B7-1, B7-2 and I-A^d proteins was analyzed by flow cytometry using appropriate primary mAb against the particular protein and a fluorescine isothyocyanate (FITC) conjugated secondary antibody. Stably transfected tumor cell lines were established, and clones were generated by limiting dilution. Cloned transfected tumor cell lines were periodically monitored for the surface expression of

the protein(s). The tumorigenicity of the transfected tumor cells was determined by in vivo tumor challenge experiments using syngeneic Balb/c mice.

(6) BODY: Experimental Results

Constitutive expression of MHC class II:

The results of the earlier experiments have shown that the expression of either B7-1 or B7-2 costimulatory molecules on both the sublines, 66.1 and 410.4, only resulted in significantly delayed tumor growth compared to the corresponding wild type tumor cells. In all these situations, it is conceivable that the B7-transfectants are able to costimulate the CD8+ effector T cells. However, the failure to see complete rejection of the transfected tumors could be due to the lack of T cell help, in the form of cytokine(s) secreted by CD4+ T cells. Therefore, it is important to activate the tumor-specific CD4+ T cells which normally recognize an antigen in the context of MHC class II molecules. Our previous study in a sarcoma tumor model showed that MHC class II+ tumor cell transfectants induced long-lasting specific anti-tumor immunity (18, 20).

The initial problems in constitutively expressing syngeneic MHC class II (I-A^d) molecules on the 66.1 and 410.4 wild type tumor cells were overcome. Two different stratategies were successfully applied. In one, higher amounts (10 ug) of plasmid vector containing the I-A^d alpha and I-A^d beta genes run by a SV40 promoter were used together with very low amounts (0.2 ug) of a separate plasmid containing neomycin resistance gene. The latter plasmid allowed selection of the transfectants that grew in G418 containing medium. In an alternate attempt, the I-A^d alpha and I-A^d beta genes were cloned into a different plasmid vector containing human beta actin promoter to drive the gene. This vector also has an additional advantage of having the neomycin resistance gene in it and therefore, cotransfection with another vector containing a drug selection marker was not necessary. The wild type 410.4 and 66.1 tumor cells were transfected with MHC class II (Ad) genes and 410.4/Ad clones and 66.1/Ad clones were generated by limiting dilution cloning of the MHC class II positive lines. Similarly, control transfectants were made by transfecting only with the plasmid containing neomycin resistant gene (410.4/Neo and 66.1/Neo). Figures 1 and 2 show the flow cytometer analysis on the expression of I-A^d molecules by 410.4/Ad and 66.1/Ad clones and control transfectants, respectively. Two different monoclonal antibodies were used to detect I-Ad, MKD6 and M5.114, and both gave similar results.

While 410.4/Ad clones and 66.1/Ad clones expressed of high levels of I-Ad molecules, the 410.4/Neo and 66.1/Neo clones did not express of I-Ad.

Immunogenicity of transfectants expressing MHC class II:

To test the effect of constitutive expression of syngeneic MHC class II (I-Ad molecules) on the growth/rejection of 410.4 tumor cells, groups of naive Balb/c mice were injected s.c. with 410.4/Ad transfectants (clones 2.24, 1.2 and 1.12). The control group of mice received 410.4/Neo transfectants. The growth of 410.4/Neo transfectants was not significantly different from that of 410.4/WT tumor cells observed in earlier experiments (Annual Report 1996, Figure 3A). This indicates that the expression of Neomycin resistant gene alone did not influence the growth of these tumor cells in vivo. The I-Ad positive clones exhibited varied growth patterns. With clone 2.24, 60% of the mice (3/5) showed no tumor growth up to 60 days and the remaining 2/5 mice developed slower but progressive tumors (Figure 3B). Clone 1.2 showed slower but progressive tumor growth in all the mice tested (Figure 3C). However, the maximum tumor growth on day 77 was much smaller compared to that of Neo transfectants. The growth of clone 1.12 was similar to the Neo transfectants (Figure 3D). All the three clones expressed similar levels of I-Ad on their surface, and hence the observed clonal variation in the in vivo growth can not be attributed to their potential ability to present MHC class II restricted tumor antigens. Other contributing factors in eliciting T cell response remain to be studied.

Similar experiments were carried out with the 66.1 cell line. The 66.1/Neo control transfectants exhibited progressive growth as observed earlier with the 66.1/WT tumor cells (Annual Report 1996, Figure 4A). The 66.1/Ad line, despite the constitutive expression of I-Ad molecules (Figure 2 panels E and F), showed no difference in the in vivo growth compared to 66.1/Neo transfectants (Figure 4B). Upon limiting dilution cloning of this line, several I-Ad positive clones as well as several I-Ad negative clones were derived. Therefore, it is possible that the progressive in vivo growth observed with the 66.1/Ad line is contributed by the population of cells that down regulated I-Ad expression rapidly. Subsequent experiments were carried out with clones derived from this line that stably expressed I-Ad, and yielded better results. Two of the 66.1/Ad positive clones tested (clones 1.11 and 2.13) showed significant delay in the onset of the solid tumor growth (Figures 4C and 4D). Palpable tumor was not seen in majority of the mice until 40-60 days after a s.c. challenge. A fraction of

mice in both groups (3/6 with clone 1.11 and 2/8 with clone 2.13) completely rejected the challenged tumor cells.

Taken together these results show that MHC class II positive transfectants (both 410.4/Ad and 66.1/Ad) are able to induce moderate level of tumor immunity, similar to that observed in earlier experiments with their B7-1 or B7-2 counterparts (Annual Reports 1995, 1996). It is suggested that the constitutive expression of MHC class II by the tumor cells facilitates activation of tumor specific CD4+ T cells which could help the CD8+ killer T cells. However, unlike the overwhelming results obtained with similar strategies in the sarcoma model (18, 20, 39), the mammary tumor cells expressing either MHC class II molecules or the B7-1 or B7-2 molecules alone did not induce complete tumor rejection in 100% of the mice.

Growth of MHC class II positive transfectants in nude mice:

To address the possible involvement of T cells in the anti-tumor immunity induced by the MHC class II positive transfectants, the transfectants were injected into athymic Balb/c nude mice and their growth was monitored. Figure 5 shows the results of the experiment. The 410.4/Ad clones 1.12 and 2.24 (Figures 5A and B), and the 66.1/Ad clone 1.11 (Figure 5C) all showed progressive growth in 100% of the nude mice. The growth kinetics was much faster compared to the growth of the individual clones in immunocompetant Balb/c mice. A palpable solid tumor was seen as early as 5-7 days after challenge which grew steadily in all the nude mice. Furthermore, unlike Balb/c mice, there was no difference in the growth of the clones 410.4/Ad-2.24 and 410.4/Ad-1.12 in nude mice. Together these results indicate that the T cells in immunocompetant Balb/c mice might play a crucial role in the induction of anti-tumor immunity by MHC class II positive tumor cells contributing to their delayed growth and in some cases complete rejection. Conversely, other components such as macrophages, natural killer cells that are intact in nude mice do not seem to contribute significantly to the early processes of tumor rejection.

Immunogenicity of mixture of 410.4/B7-1 and 410.4/Ad tumor cells:

Earlier experiments showed that gene-modified tumor cells expressing either of the T cell costimulatory molecules B7-1 or B7-2 induced moderate level of tumor immunity in immunocompetant mice (Annual Reports 1995, 1996). In the present

report it is shown that tumor cells expressing the syngeneic MHC class II, I-Ad, induced moderate level of tumor immunity in immunocompetant mice. It is envisaged that in both situations the anti-tumor immunity is accomplished by activating tumor-specific CD8+ and CD4+ T cells, respectively. It was of interest to test whether it is possible to activate both CD4+ and CD8+ T cells by injecting a mixture of the two types of transfected tumor cells. Simultaneous activation of both T cell subsets might enhance the anti-tumor immunity. Accordingly, Balb/c mice were injected with a mixture of transfectants containing equal numbers of 410.4/B7-1 and 410.4/Ad tumor cells. As shown in Figure 6, the tumor grew slow in about 50% of the mice while the rest of the mice showed faster tumor growth. One possible explanation for the failure to see an increased immunogenicity with mixed cells is that the maximal activation of CD4+ T cells require both signal 1 (via MHC class II) and signal 2 (via B7-1) at the same time. It is possible that such simultaneous signalling could be delivered only when both MHC class II and B7-1 molecules are present on the same cell. This would mean that signalling through the T cell receptor and the CD28 receptor (the appropriate ligands being MHC class II and B7-1, respectively) may perhaps occur in a polarized fashion on the T cell surface. This possibility could be addressed by using tumor cells that coexpress both MHC class II and B7-1 molecules (see below). However, it is intriguing that the moderate immunity that was seen when these transfectants were injected separately seems to be lost when the mixture of cells was given.

Can irradiated MHC class II transfectants induce protective immunity?:

Previous experiments showed that multiple injections of irradiated 66.1/B7-1 tumor cells, but not 66.1/WT cells, induced protective immunity in 5/10 mice against subsequent challenge with live 66.1/WT tumor cells (Annual Report 1996). Therefore, it was of interest to see whether multiple injections of MHC class II transfected tumor cells will protect the host against future challenge with wild type tumor cells. The previously standardized irradiation dose (3000 rad) was used to irradiate the tumor cells. Unprimed mice and mice that were primed with multiple injections of irradiated 410.4/WT tumor cells were susceptible to future challenge with live 410.4/WT tumor cells (Table 1). By contrast, mice that were primed with multiple injections of irradiated 410.4/Ad tumor cells (clones 2.24 and 1.12) were completely protected against future challenge with live 410.4/WT tumor cells (Table 1). It is possible that irradiated 410.4/Ad tumor cells are able to function as APC and directly present tumor antigen to the infiltrating CD4+ T cells. In addition, irradiated tumor cells may initiate a local

inflammatory response which would recruit other professional APC which may pick up tumor antigens from dying tumor cells and further continue the APC function, a process refered to as cross priming (54). It is interesting to note that although the clones 410.4/Ad-2.24 and 410.4/Ad-1.12 when given as live cells exhibited different growth patterns (Figures 3B and 3D), upon irradiation they were equally capable of immunizing the host.

When similar experiments were carried out with 66.1 cell line different results were obtained. Multiple injections of irradiated 66.1/Ad (clone 1.11) tumor cells did not protect the mice against subsequent challenge with live 66.1/WT tumor cells, although there was a slight delay in the onset of challenge tumor growth (Table 2). This difference in the immunizing capacities between 410.4/Ad and 66.1/Ad tumor cells can be attributed, at least in part, to the innate immunogenicity of the 410.4 cell line. On the other hand, multiple injections of irradiated 66.1/B7-1 tumor cells were shown to protect at least 50% of the mice against future challenge with wild type tumor cells (Annual Report 1996). To clarify this discrepancy, additional experiments with other 66.1/Ad clones need to be performed.

Interleukin 12 (IL-12) synergizes with B7-1 and induces potent anti-tumor immunity:

The failure to induce complete rejection of B7-1 or B7-2 transfectants of 410.4 and 66.1 tumor cell lines suggests that costimulation of CD8+ T cells alone is certainly not sufficient to maximally activate the tumor-specific effector T cells. The limiting factor(s) could be lack of T cell help (e.g. CD4+ T cells) (22) and/or cytokines that might amplify the T cell functions (e.g. IL-12) (50). The possible help from the activated CD4+ T cells to mediate tumor rejection is discussed below. In vitro studies showed that IL-12 in combination with B7 induced enhanced activation of T cells (51, 52). IL-12 is a very potent cytokine and has been used to induce regression/rejection of a variety of tumors (53). Therefore, it was of interest to investigate whether administration of IL-12 would further enhance the anti-tumor immunity induced by B7-transfected tumor cells. Part of the results of this experiment was reported previously (Annual Report 1996), and the results of the complete experiment with additional controls is presented here. Groups of Balb/c mice were injected s.c. with 410.4/WT or 401.4/B7-1 (clone 11) tumor cells. Each group was divided into two subgroups, and while one subgroup was left untreated the other subgroup received 9 daily injections of recombinant murine IL-12. Figure 7 shows the primary tumor growth in these mice.

Administration of IL-12 resulted in significant reduction in the growth of 410.4/WT tumor cells (Figures 7A and B), although none of the animals completely rejected the wild type tumor. By contrast, 410.4/B7-1 (clone 11) tumor cells showed a significant delay in the growth compared to that of wild type tumor cells (Figures 7A and C). Administration of IL-12 induced complete regression of 410.4/B7-1 tumor cells, although in 2/5 mice a small solid tumor grew but later regressed (Figure 7D). These results show that while IL-12 alone could significantly reduce the growth of the wild type tumor cells, IL-12 has a profound effect when the tumor cells express B7-1 molecules. Presumably, costimulation of CD8+ T cells and selective proliferation of Th1 type CD4+ T cells (induced by IL-12) together seem to effect complete tumor regression.

To further study the synergistic effect of IL-12 and B7-1, the mice that received 410.4/B7-1 tumor cells, with or with out IL-12, were challenged i.v. with live 410.4/WT tumor cells. These mice were later sacrificed and examined for metastasis of tumor into lungs, liver and spleen. The control group of mice received an i.v. challenge of 410.4/WT tumor cells alone without any prior immunization. The unimmunized control mice and the mice that were previously immunized with 410.4/B7-1 cells alone showed a lot of metastatic nodules in lung and liver. This is also indicated by the increase in total weight of the lungs and liver in these mice. By contrast, the mice that were immunized with 410.4/B7-1 cells and IL-12 did not show any visible metastatic nodules in lung or liver (Table 3). All mice showed enlarged spleen (splenomegaly). The 410.4 tumor cells are known to secrete granulocyte/macrophage colony stimulating factor which could be attracting a lot of cellular infiltration. This aspect has to be further investigated. In any event, prior immunization with 410.4/B7-1 cells combined with IL-12 administration has induced potent immunity that prevented metastasis following subsequent challenge with wild type tumor cells.

It is very encouraging to see that a combination of B7-1 and IL-12 resulted in enhanced tumor immunity that can not only cause rejection of local tumor growth but also has a profound effect in preventing metastasis following i.v. inoculation of fairly large numbers of wild type tumor cells. It will be very interesting to see the effect of combining IL-12 administration with 66.1/B7 and 66.1/Ad transfectants because the subline 66.1 is known to readily spontaneously metastasize to lung and liver. In addition, studies on the role of IL-12 in therpeutic treatment of previously established tumor will be very useful and could be translated into clinical use.

Generation of tumor cells coexpressing MHC class II and B7-1 molecules:

It is evident from the experiments described above and the previous reports (Annual Reports 1995 and 1996) that injection of live 410.4 and 66.1 tumor cell transfectants expressing B7-1, B7-2 or I-Ad molecule induced only a moderate immunity. At the best, complete rejection was seen in some mice but not in all. Injection of a mixture of B7-1 expressing cells and I-Ad expressing cells also failed to induce rejection. This suggested that perhaps coexpression of I-Ad and B7-1 molecules by the tumor cells will help activate both CD4+ and CD8+ T cells simultaneously and lead to a vigorous elicitation of anti-tumor immunity. Our previous report in a sarcoma model supports this contention (40).

Towards this goal, clones of 410.4/Ad and 66.1/Ad transfectants were used to super transfect the B7-1 gene. Since these transfectants are already G418 resistant, they have to be cotransfected with another drug marker in order to allow selection of the transfectants expressing both I-Ad and B7-1 molecules. Initial attempts with hygromycin resistant gene and methotrexate resistant gene, both of which have been used in different tumor cell lines, were unsuccessful. After an empirical titration of the drugs for sensitivity, it was difficult to determine a drug concentration that could be used to select the double transfectants and at the same time kill the zeocin-untransfected tumor cells. However, another drug marker, zeocin, proved helpful. Accordingly, 410.4/Ad and 66.1/Ad clones have been supertransfected with murine B7-1 gene together with a plasmid containing zeocin resistant gene. The double transfectants were grown in media containing both G418 and zeocin. Positive lines were subsequently cloned by limiting dilution and clones expressing both I-Ad and B7-1 molecules have been derived. Several 410.4/Ad/B7-1 and 66.1/Ad/B7-1 clones have been established. Flow cytometer analysis revealed coexpression of MHC class II (I-Ad) and B7-1 molecules by these transfected tumor cells. Figures 8 and 9 depict a few representatives of the 410.4/Ad/B7-1 and 66.1/Ad/B7-1 clones, respectively. Although the clones are clearly positive for both I-Ad and B7-1 molecules, many of them seem to express a lot more I-Ad than B7-1. It is not clear at this point what is causing this difference since many factors could be responsible including the promoters used, site of integration in the host chromosome, intergenic regulation, etc. Nonetheless, their level of expression will be monitored periodically.

The immunogenicity of the tumor cells coexpressing MHC class II and B7-1 molecules will be studied by injecting them into syngeneic Balb/c mice and following the tumor growth in vivo. It is anticipated that these transfectants will be able to activate both CD4+ T cells and CD8+ T cells resulting in strong anti-tumor immunity. In addition, experiments will be performed to study the metastatic potential of the tumor cells expressing both MHC class II and B7-1. Furthermore, these transfectants will be used in therapeutic experiments to treat preexisting tumors. All these experiments are under way.

(7) CONCLUSIONS:

The results of the experiments presented here show that constitutive expression of syngeneic MHC class II molecules (I-Ad) by the mammary tumor cell lines, 410.4 and 66.1, enabled them to induce moderate immunity resulting in a significantly slower growth in vivo in naive Balb/c mice compared to the wild type counterparts or the control transfectants (410.4/Neo and 66.1/Neo). In some cases, a proportion of the mice showed complete rejection of the I-Ad positive tumor cells (66.1/Ad clones). This is very similar to the effects seen with the transfected tumor cells expressing the T cell costimulatory molecules B7-1 or B7-2. Together these findings suggest that these genemodified tumor cells activate the tumor-specific CD4+ T cells and CD8+ T cells, respectively. Rapid growth of the I-Ad positive tumor cells in the immunocompromised nude Balb/c mice further substantiates the crucial role played by the T cells in inducing anti-tumor immunity in immunocompetant Balb/c mice. Conversely, since the nude mice do not lack macrophage and natural killer cell functions, it seems that these non-T cell effectors do not play a role at least in the initial phase of the development of tumor immunity.

Multiple injections of irradiated 410.4/Ad tumor cells, not irradiated 410.4/WT tumor cells, induced protective immunity in 100% of the mice against subsequent challenge with wild type tumor cells. This is even better than the partial protection seen with the multiple injections of irradiated 66.1/B7-1 transfectants. Collectively, these studies demonstrate that growth arrested immunogenic tumor cells expressing B7-1 or MHC class II molecules can be used to induce tumor immunity. The obvious advantage of such gowth arrested vaccine tumor cells may be preferable over their live counter parts in possible clinical use. However, the mechanism by which the irradiated cells induce tumor immunity remain largely unknown. It is quite likely that both direct presentation of the relevant tumor antigen by the genemodified tumor cells

as well as indirect presentation of such antigens by host derived APC may be contributing to the observed immunity.

The synergistic effect seen with a combination of IL-12 administration and priming naive mice with B7-1 transfected tumor cells is very appealing. The combination treatment is certainly superior to the treatment with IL-12 alone in controlling primary tumor growth. Furthermore, the profound effect of priming with B7-1 transfectants plus IL-12 administration in controlling metastasis after i.v. challenge of wild type tumor provides evidence for adapting this strategy in therapeutic applications. Further studies need to be carried out to compare the combination procedures to treat mice bearing established wild type tumors. Informations derived from those experiments would give insights into possible clinical use.

The idea that a simultaneous activation of both CD4+ and CD8+ T cells will help inducing a strong and vigorous anti-tumor immunity is very attractive. The failure to see any beneficial effect by using mixture of MHC class II expressing tumor cells and B7-1 expressing tumor cells indicates that these two molecules need to be present on the same cell. Towards this end, both 410.4 and 66.1 tumor cells coexpressing I-Ad and B7-1 molecules have been derived. Initial experiments are under way to determine the immunogenicity of these double transfectants. It is anticipated that these double transfectants may be readily rejected by naive Balb/c mice. Subsequent experiments will involve the assessment of metastatic potential of these double transfectants, their ability to induce protective immunity and to control/cure preestablished tumors. Furthermore, analysis of the T cell populations induced by these immunogenic tumor cells, comparison of cytokines produced by the tumor activated T cells and the regulatory mechanisms involved in deciding the elicitation of useful tumor-specific immune response will be carried out.

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FIGURE LEGENDS

Figure 1:

Flow cytometry analysis of a control transfectant clone (401.4/Neo - panels A' and B') and three representative 410.4/Ad clones (clone-1.2 - panels A and B; clone-2.24 - panels C and D; and clone-1.12 - panels E and F) are shown. The tumor cells were stained with either mouse anti-rat-FITC alone (left panels) or stained with a mouse mAb against murine I-A^d (MKD6) and then with the goat anti-mouse-FITC conjugate (right panels). The y axes indicate the relative number of cells and the x axes indicate log fluorescence intensity. Staining with a rat mAb against I-A^d (M5.114) showed comparable results (data not shown).

Figure 2:

Flow cytometry analysis of a control transfectant clone (66.1/Neo - panels A' and B') and three representative 66.1/Ad clones (clone-1.11 - panels A and B; clone-2.13 - panels C and D; and 66.1/Ad Line - panels E and F) are shown. The tumor cells were stained with either mouse anti-rat-FITC alone (left panels) or stained with a rat mAb against murine I-A^d (M5.114) and then with the mouse anti-rat-FITC conjugate (right panels). The y axes indicate the relative number of cells and the x axes indicate log fluorescence intensity. Staining with a mouse mAb against I-A^d (MKD6) showed comparable results (data not shown).

Figure 3:

Tumorigenicity of 410.4/Ad positive tumor cells. Naive Balb/c mice received 3 x 10e5 (e = exponential) live tumor cells s.c. on day 0. The control group received 410.4/Neo transfectants (panel A). The other groups received 401.4/Ad clone - 2.24 (panel B), clone - 1.2 (panel C) or clone - 1.12 (panel D). Tumor growth was monitored as described in the text. Each line represents an individual mouse.

Figure 4:

Tumorigenicity of 66.1/Ad positive tumor cells. Naive Balb/c mice received 3 x 10e5 (e = exponential) live tumor cells s.c. on day 0. The control group received 66.1/Neo transfectants (panel A). The other groups received 66.1/Ad Line (panel B), clone - 1.11 (panel C) or clone - 2.13 (panel D). Tumor growth was monitored as described in the text. Each line represents an individual mouse.

Figure 5:

Tumor growth in nude mice. Groups of naive Balb/c nude mice received s.c. 3 x 10e5 live tumor cells on day 0. 410.4/Ad clone - 1.12 (panel A), 410.4/Ad clone - 2.24 (panel B) and 66.1/Ad clone - 1.11 (panel C). Tumor growth was monitored as described in the text. Each line represents an individual mouse.

Figure 6:

Mixed tumor cells challenge. Naive Balb/c mice received s.c. 410.4/B7-1 (clone 11) and 410.4/Ad (clone 1.2) tumor cells, 1.5 x 10e5 each on day 0. Tumor growth was monitored as described in the text. Each line represents an individual mouse.

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Figure 7:

IL-12 enhances B7-1-mediated regression of primary tumor growth. Groups of naive Balb/c mice received s.c. 3 x 10e5 live 410.4/WT (panels A and B) or 410.4/B7-1 positive (panels C and D) tumor cells on day 0. Each group was divided into two subgroups. One subgroup received IL-12 as mentioned in the text (panels C and D) and the other subgroup did not (panels A and B). Tumor growth was monitored as described in the text. Each line represents an individual mouse.

Figure 8:

Flow cytometry analysis of 401.4 transfectants coexpressing I-Ad and B7-1 molecules. The following transfectants: 410.4/Neo (panel A) and three representative 410.4/Ad/B7-1 clones: clone - 19 (panel B), clone - 40 (panel C) and clone - 41 (panel D) are shown. The tumor cells were stained with mouse anti-rat-FITC alone (thin line) or stained with the either of the two primary antibodies: a rat mAb against murine I-A^d (M5.114) (dotted line) or with a rat mAb against murine B7-1 (1G10) (thick line). Both these latter preparations were subsequently stained with a secondary Ab, the mouse anti-rat-FITC conjugate. The y axes indicate the relative number of cells and the x axes indicate log fluorescence intensity.

Figure 9:

Flow cytometry analysis of 66.1 transfectants coexpressing I-Ad and B7-1 molecules. The following transfectants: 66.1/Neo (panel A) and three representative 66.1/Ad/B7-1 clones: clone - A3 (panel B), clone - 49 (panel C) and clone - 35 (panel D) are shown. The tumor cells were stained with mouse anti-rat-FITC alone (thin line) or stained with the either of the two primary antibodies: a rat mAb against murine I-A^d

(M5.114) (dotted line) or with a rat mAb against murine B7-1 (1G10) (thick line). Both these latter preparations were subsequently stained with a secondary Ab, the mouse anti-rat-FITC conjugate. The y axes indicate the relative number of cells and the x axes indicate log fluorescence intensity.

Table 1

Multiple Injections of Irradiated 410.4/Ad Transfectants Induced Protective Immunity

Immunization Tumor cells	Dose	Challenge Tumor (Dose)	Tumor Incidence	Tumor Onset (No. of Days)
None		410.4/WT (3×10^5)	5/5	7-14
410.4/WT	4X 1 x 10^6	410.4/WT (3×10^5)	5/5	7-14
410.4/Ad (Clone 2.24)	4X 1 x 10^6	410.4/WT (3×10^5)	0/6	>120
410.4/Ad (Clone 1.12)	4X 1 x 10^6	410.4/WT (3×10^5)	0/5	>120

Balb/c mice received four weekly i.p.injections of irradiated (3300 rad) tumor cells as indicated. Control group did not receive any immunization. All mice were challenged s.c. with 3×10^5 live 410.4/WT tumor cells. Immunized mice were challenged 35 days after last immunizing injection. Tumor incidence was scored at the end of the observation period and depicted as the number of mice with solid tumor in the flank/total number of mice challenged.

Table 2

Multiple Injections of Irradiated 66.1/Ad Transfectants Fail to Induce Protective Immunity

Immunization Tumor cells	Dose	Challenge Tumor (Dose)	Tumor Incidence	Tumor Onset (No. of Days)
None		66.1/WT (3×10^5)	5/5	7-14
66.1/WT	4X 1×10^6	66.1/WT (3×10^5)	5/5	7-14
66.1/Ad (clone 1.11)	4X 1×10^6	66.1/WT (3×10^5)	5/5	14-21

Balb/c mice received four weekly i.p.injections of irradiated (3300 rad) tumor cells as indicated. Control group did not receive any immunization. All mice were challenged s.c. with 3×10^5 live 66.1/WT tumor cells. Immunized mice were challenged 28 days after last immunizing injection. Tumor incidence was scored at the end of the observation period and depicted as the number of mice with solid tumor in the flank/total number of mice challenged.

Table 3

IL-12 synergizes with B7-1 and prevents experimental metastasis

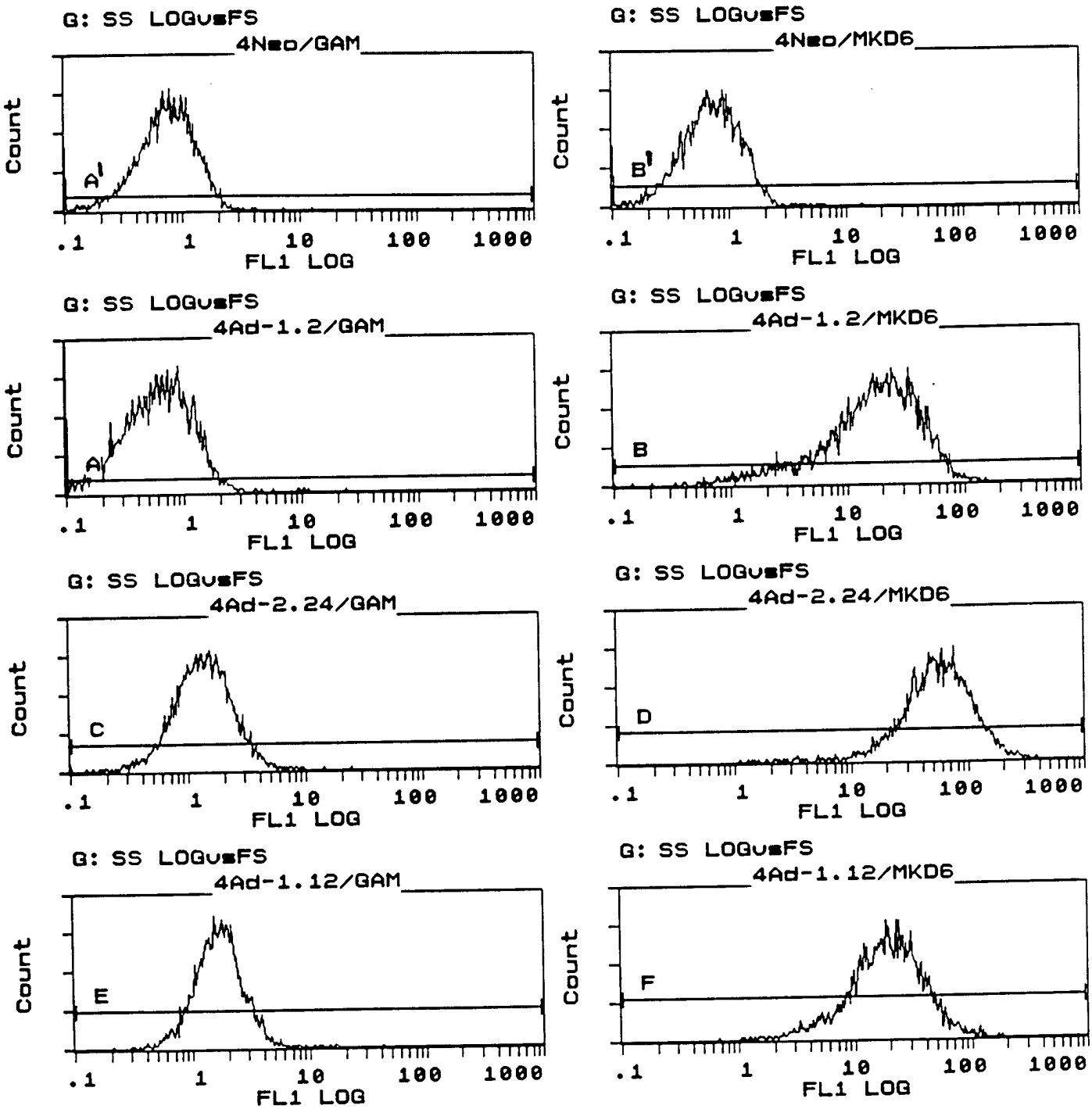
Priming (s.c.)	IL-12 (i.p.)	Challenge (i.v.)	Lung weight in mg (mets)[a]	Liver weight in mg (mets)[b]
None	-	410.4/WT	643, 597,750, 775 (++++)	896, 947, 1109, 1210 (++)
410.4/B7-1	-	410.4/WT	710, 650, 680, 725, 583 (+++)	1115, 1025, 990, 1105, 670 (+)
410.4/B7-1	+	410.4/WT	270, 308, 347, 261, 307 (--)	870, 930, 780, 650, 715 (--)

Mice were either unprimed or primed s.c. with 3 x 10^5 live 410.4/B7-1 (clone 11) tumor cells alone or mixed with IL-12 (0.25 microgram/injection). The IL-12 treatment was followed by daily i.p. injections for subsequent 8 days. Thirty days later all mice were challenged i.v. with 1 x 10^6 live wild type tumor cells. The weight of lungs and liver of individual mouse is depicted. The data in parantheses represent average number of mets in each group.

a Mice were sacrificed and examined for mets in lungs and liver when they became moribund. Metastatic nodules in lungs were scored as follows: -- = No visible mets, + = 1-10 mets, ++ = 10-50 mets, +++ = 50-100 mets and ++++ = >100 mets.

b Metastatic nodules were not as clearly visible as in lungs except for the large nodules. Metastatic nodules in liver were scored as follows: -- = No visible mets, + = 1-5 mets, ++ = > 5 mets.

Figure 1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1
10
100
1000
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G: SS LOGvsFS
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Count
B'
.1
1
10
100
1000
FL1 LOG
G: SS LOGvsFS
4Ad-1.2/GAM
Count
A
.1
1
10
100
1000
FL1 LOG
G: SS LOGvsFS
4Ad-1.2/MKD6
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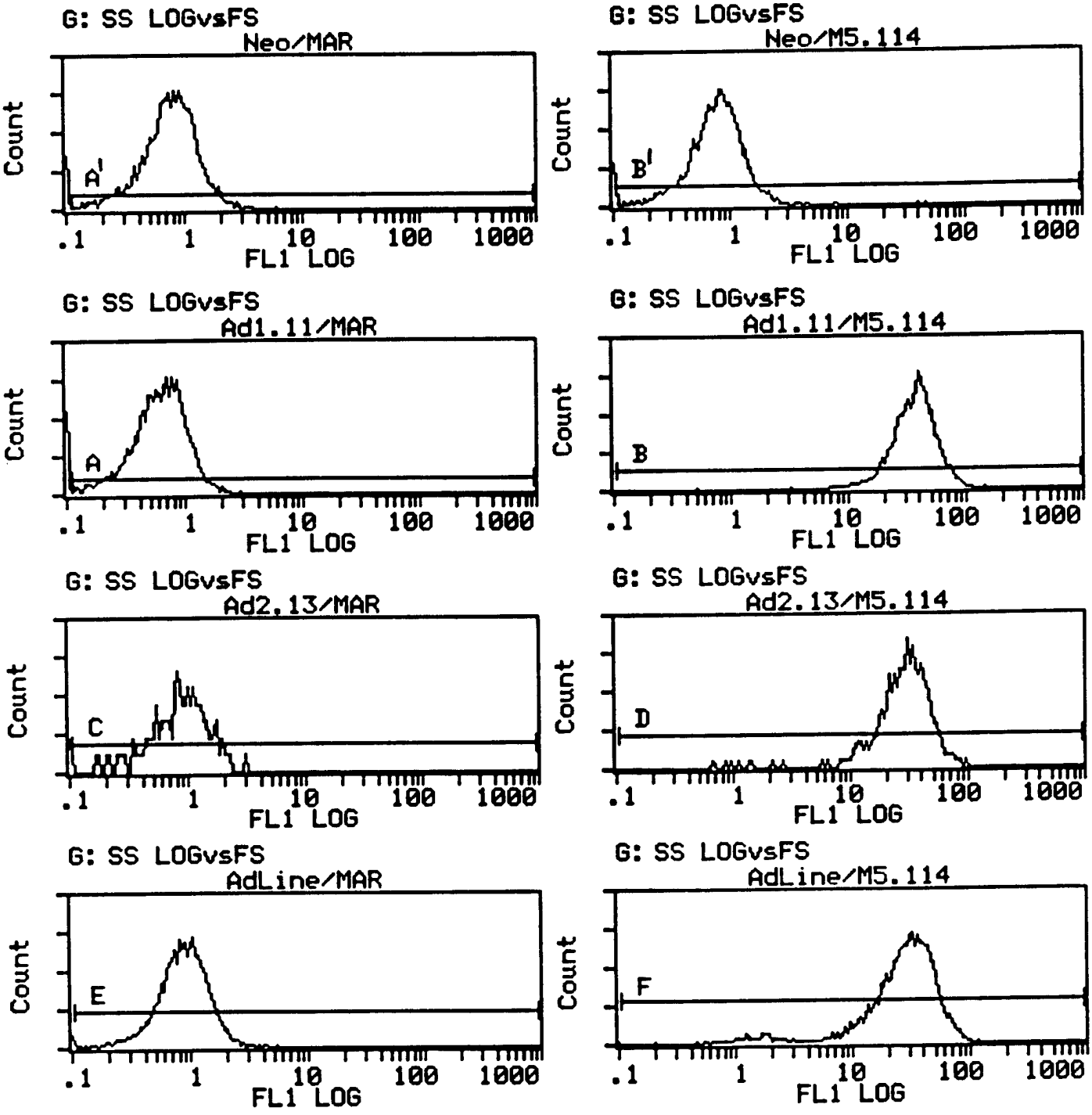
10
100
1000
FL1 LOG

Figure 2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Neo/MAR
Count
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10
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Count
E
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1
10
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Count
F
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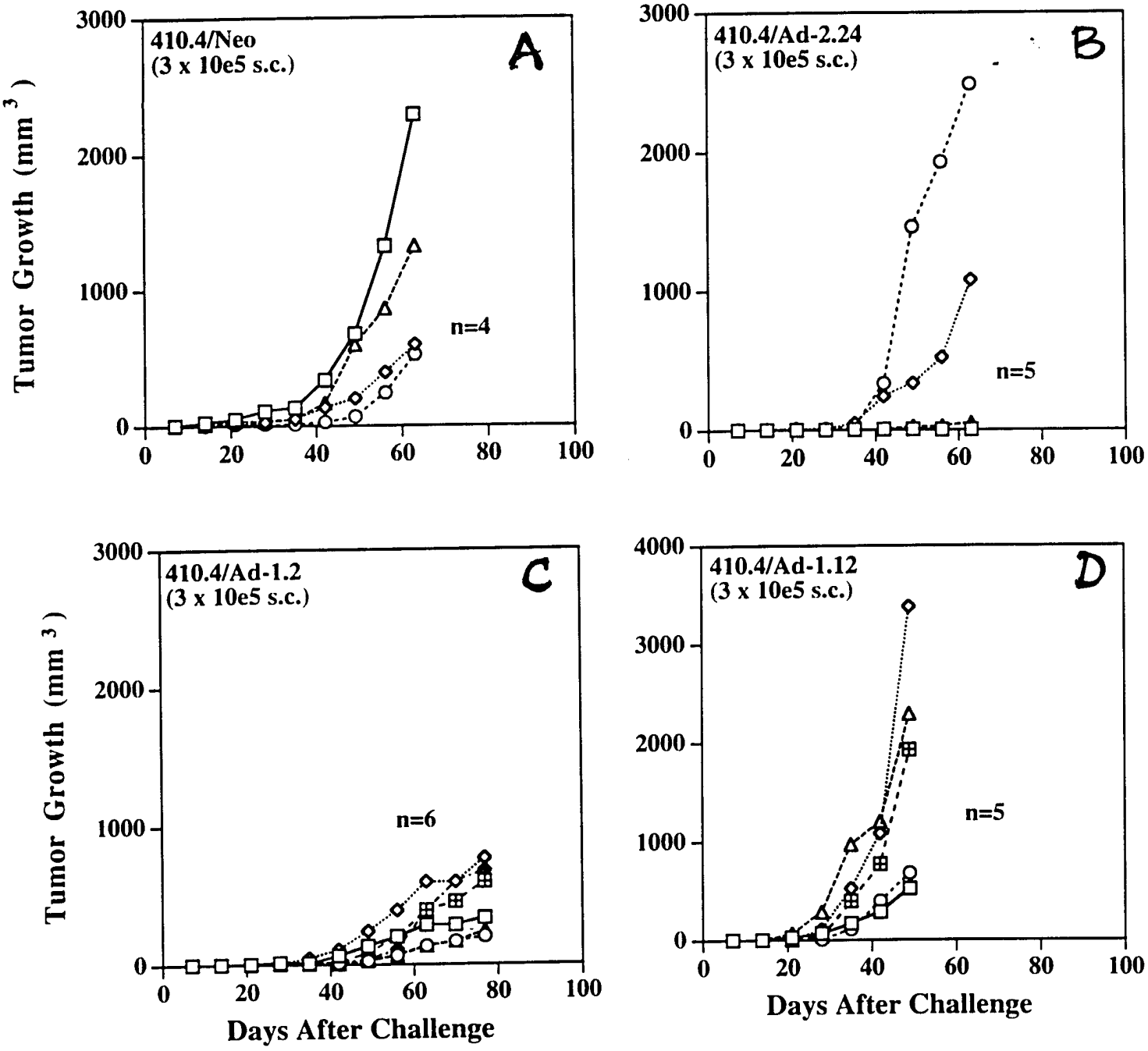
1
10
100
1000
FL1 LOG

Figure 3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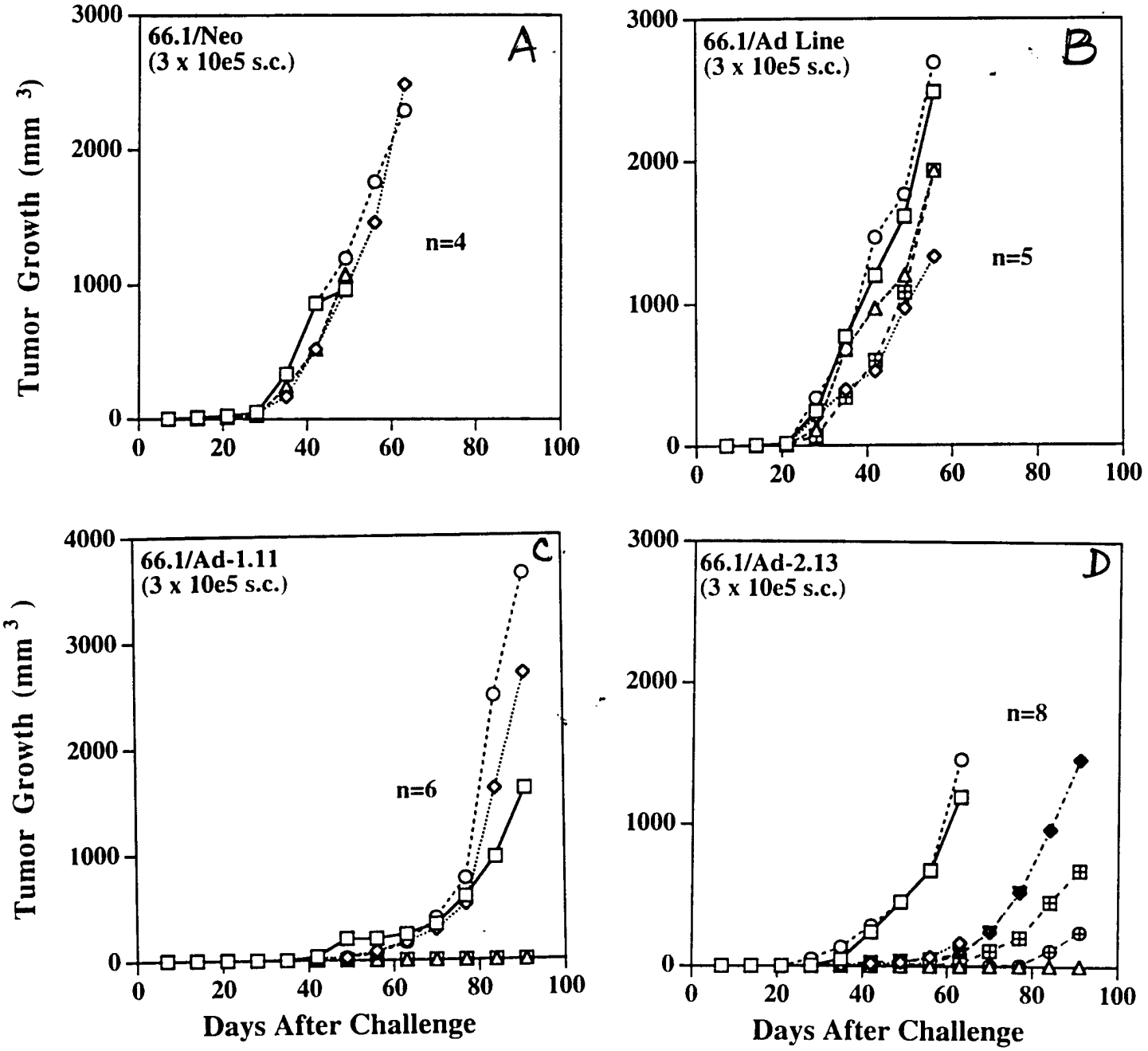
410.4/Ad-2.24
(3 x 10e5 s.c.)
B
n=5
410.4/Ad-1.2
(3 x 10e5 s.c.)
C
n=6
410.4/Ad-1.12
(3 x 10e5 s.c.)
D
n=5
Tumor Growth (mm 3)
Days After Challenge
0
20
40
60
80
100
1000
2000
3000
4000

Figure 4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66.1/Ad Line
(3 x 10e5 s.c.)
B
n=5
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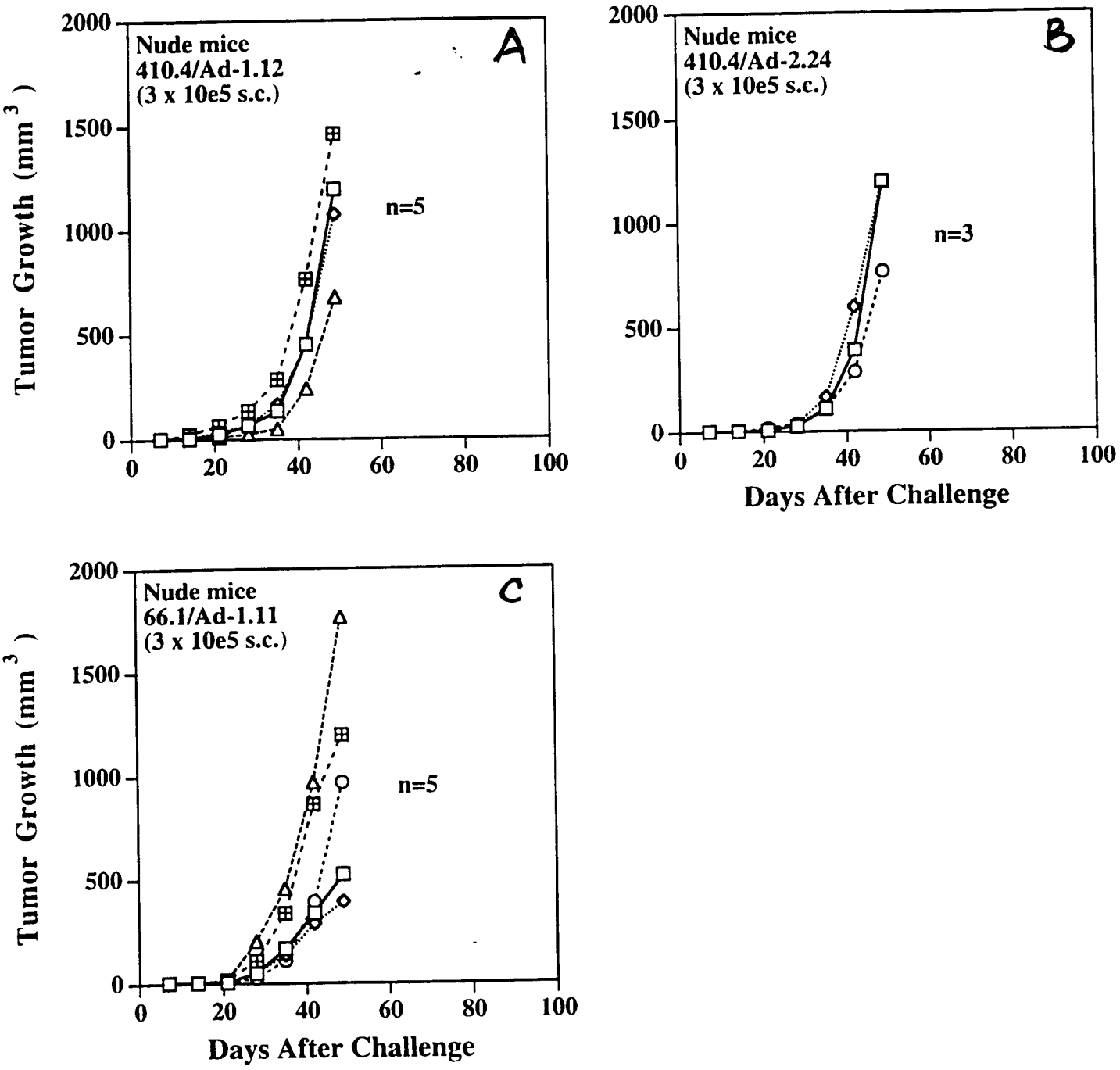
(3 x 10e5 s.c.)
C
n=6
66.1/Ad-2.13
(3 x 10e5 s.c.)
D
n=8
Tumor Growth (mm 3)
Tumor Growth (mm 3)
Days After Challenge
Days After Challenge

Figure 5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n=5
B
Nude mice
410.4/Ad-2.24
(3 x 10e5 s.c.)
n=3
C
Nude mice
66.1/Ad-1.11
(3 x 10e5 s.c.)
n=5
Tumor Growth (mm3)
Days After Challenge
0
500
1000
1500
2000
0
20
40
60
80
100

Figure 6

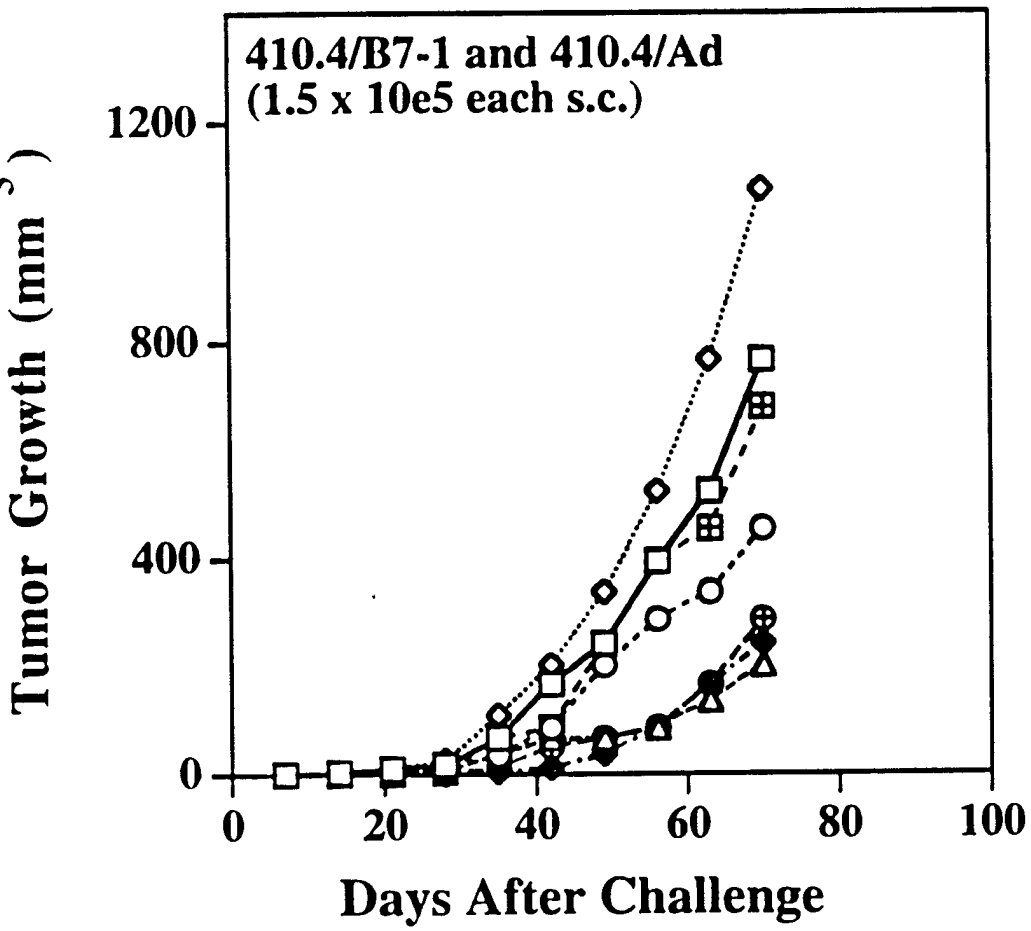

410.4/B7-1 and 410.4/Ad
(1.5 x 10e5 each s.c.)
Tumor Growth (mm 3)
1200
800
400
0
0
20
40
60
80
100
Days After Challenge

Figure 7

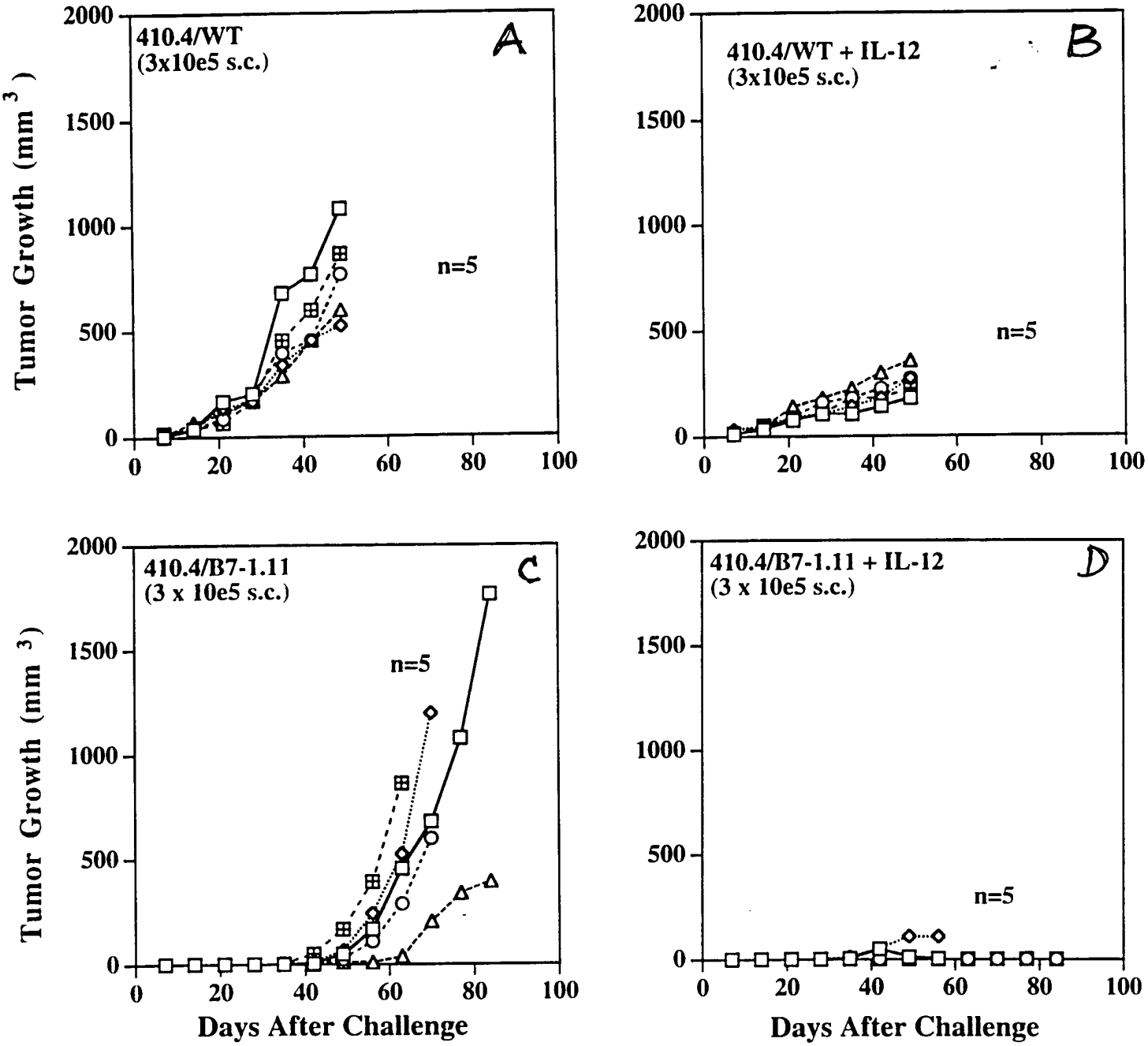
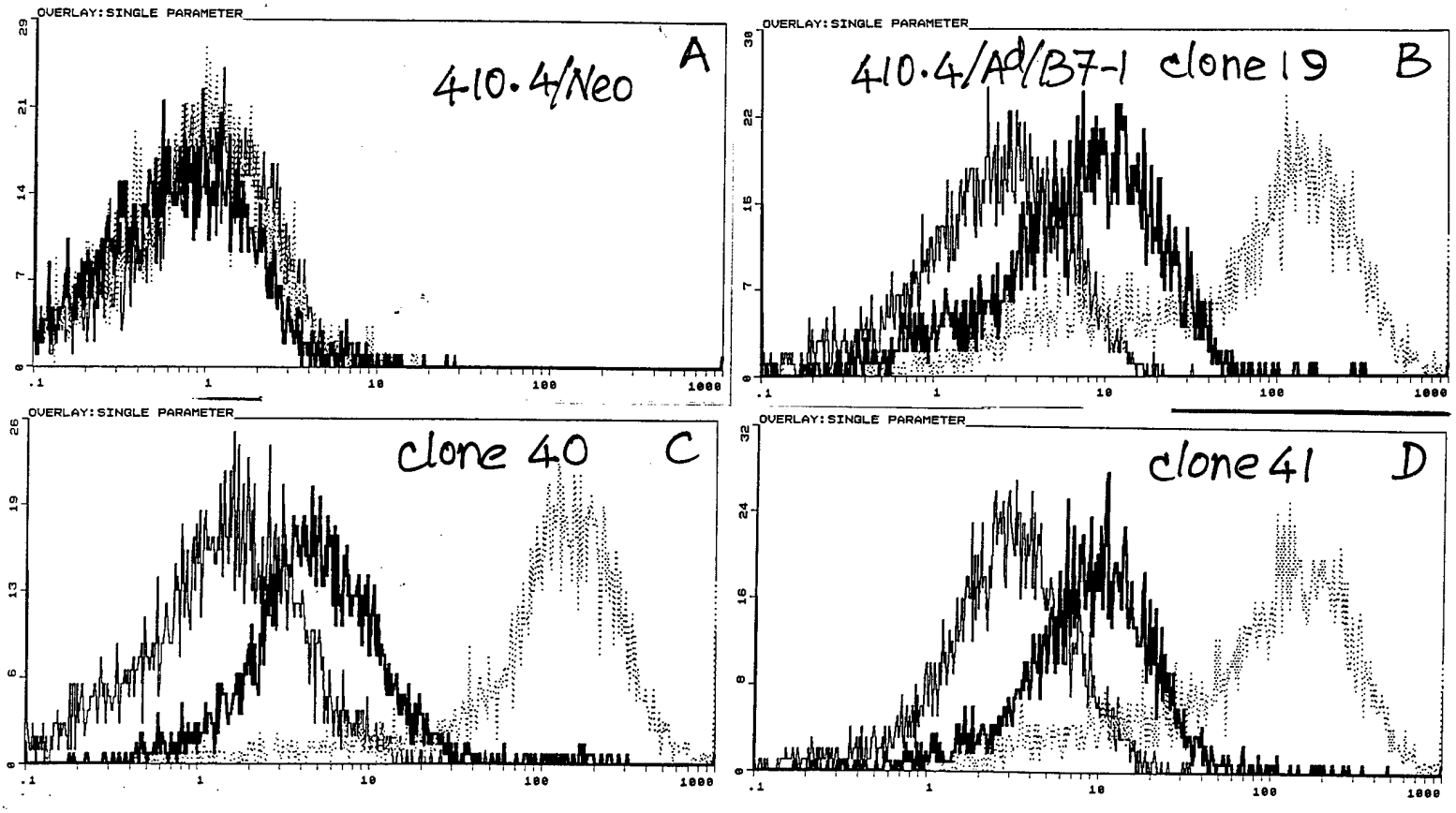

410.4/WT
(3x10e5 s.c.)
A
n=5
410.4/WT + IL-12
(3x10e5 s.c.)
B
n=5
410.4/B7-1.11
(3 x 10e5 s.c.)
C
n=5
410.4/B7-1.11 + IL-12
(3 x 10e5 s.c.)
D
n=5
Tumor Growth (mm 3)
Days After Challenge
0
500
1000
1500
2000
0
20
40
60
80
100

Figure 8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410.4/Neo
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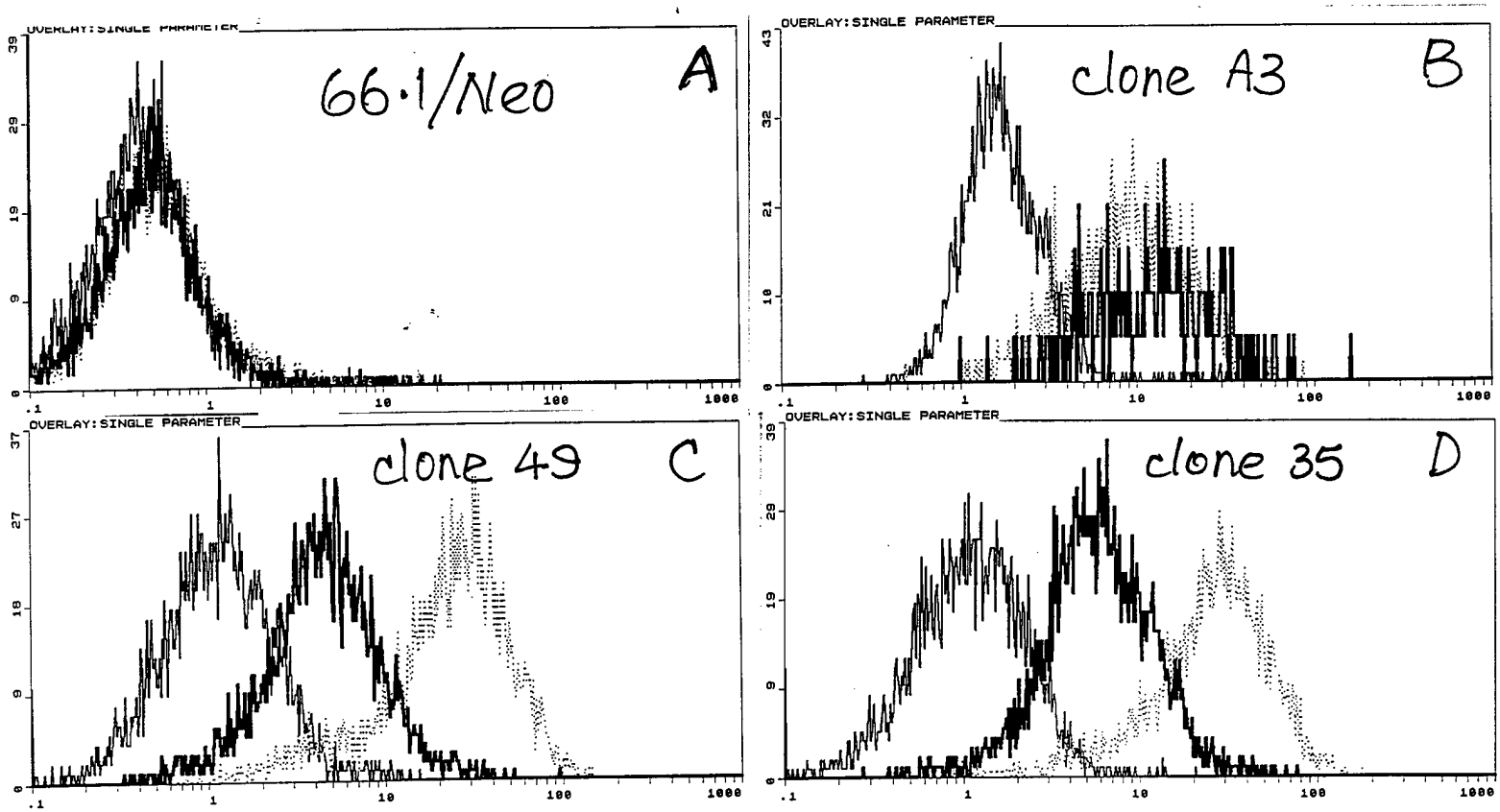
410.4/Ad/B7-1 clone 19
B
OVERLAY:SINGLE PARAMETER
clone 40
C
OVERLAY:SINGLE PARAMETER
clone 41
D

Figure 9


OVERLAY: SINGLE PARAMETER
66·1/Neo
A
OVERLAY: SINGLE PARAMETER
clone A3
B
OVERLAY: SINGLE PARAMETER
clone 49
C
OVERLAY: SINGLE PARAMETER
clone 35
D